ISTITUTO ZOOPROFILATTICO SPERIMENTALE DELLE VENEZIE

EUROPEAN UNION REFERENCE LABORATORY FOR AVIAN INFLUENZA AND NEWCASTLE DISEASE

SOP VIR 1006

MOLECULAR PATHOTYPING OF ORTHOAVULAVIRUS JAVAENSE (OAV-J) BY REAL-TIME RT-PCR AND SANGER SEQUENCING

(Fortin et al., 2023)

Standard operating procedure used by the EURL for AI and ND at the Istituto Zooprofilattico Sperimentale delle Venezie. Released on 14/10/25.

The content of this document is subject to modification without prior notice.

The commercial products referenced in the SOP are the ones currently in use at the EURL for AI/ND. Their inclusion is intended exclusively to support diagnostic laboratories in the implementation of the protocol and does not constitute any form of commercial promotion. The use of alternative reagents or instruments that prove fit for the intended purpose is not restricted under any circumstances.

Revision history

Revision	Date	Description
ed. 00	October 2025	Initial release

Contents

1. Purpose and field of application

This protocol describes the procedure to discriminate between virulent and avirulent *Orthoavulavirus javaense* (OAV-J) in biological samples in dual mode, i.e. by real-time reverse transcription polymerase chain reaction (RRT-PCR) and Sanger sequencing.

The protocol based on the assay developed by Fortin et al. (2023) consists of an array of three RRT-PCRs that employ three oligonucleotide sets with pathotype-specific probes targeting the F0 cleavage site (CS) of class I and class II OAV-J. Oligonucleotides of SET A target virulent and avirulent class II viruses of genotypes I-IV, IX-XI (including vaccine strains); oligonucleotides of SET B target virulent and avirulent class II viruses of genotypes V-VIII, XII-XXI; oligonucleotides of SET C target class I avirulent OAV-J. The three RRT-PCR reactions (sets A-C) have to be performed in parallel. However, in investigations where the circulating virus is already defined (e.g., secondary spread of a confirmed outbreak), laboratories may elect to run only the corresponding set of oligonucleotides. If pathotype confirmation by sequencing of the CS of the fusion protein gene is required, the size of the RRT-PCR amplification products consents downstream Sanger sequencing.

The procedure can be applied to RNA purified from AIV isolates, organ/tissue homogenates, stool and swabs properly collected and preserved.

The RRT-PCRs array is a downstream methods for OAV-J confirmed cases. Pathotyping based on RRT-PCR readout (i.e. based on pathotype-specific probes) is intended to reduce turnaround time in diagnostic laboratories i) to quickly confirm a strong clinical suspicion of Newcastle Disease (ND), ii) in the event of suspected secondary outbreaks linked to notified ND cases for which the CS sequence was obtained. Importantly, pathotyping confirmation by Sanger sequencing of the RRT-PCR amplicon(s) is mandatory for primary ND outbreaks (i.e. the index case), while is it highly recommended to interpret doubtful RRT-PCRs results and in case of co-infection with virulent and avirulent OAV-J (see paragraph 6.2).

2. References

- A. Fortin, A. Laconi, I. Monne, S. Zohari, K. Andersson, C. Grund, M. Cecchinato, M. Crimaudo, V. Valastro, V. D'Amico, A. Bortolami, M. Gastaldelli, M. Varotto, Newcastle Disease Collaborating Diagnostic Group, C. Terregino, V. Panzarin. A novel array of real-time RT-PCR assays for the rapid pathotyping of type I avian paramyxovirus (APMV-1). J Virol Methods 114813, 2023. doi: 10.1016/j.jviromet.2023.114813;
- Commission Decision 2006/437/EC approving a Diagnostic Manual for avian influenza as provided for in Council Directive 2005/94/EC;
- WOAH - World Organization for Animal Health, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.10, Newcastle disease (infection with Newcastle disease virus) (Version adopted in May 2021);
- SOP VIR 1000 - Sample preparation and nucleic acids isolation for the detection and typing of Avian influenza virus and Avian Orthoavulavirus type 1 by molecular methods.

3. Safety

Individual laboratories are responsible for ensuring that all the procedures described in this document are conducted under high safety standards, including awareness on chemical and biological risks. According to the risk assessment, either BSL2 or BSL3 facilities must be used. Safety rules at individual laboratories must be agreed with the biosecurity and biosafety officer and acknowledged by all the staff members involved.

4. Materials

4.1. Reagents

For storage conditions of commercial products, refer to the manufacturer's instructions.

- Nuclease-free water;
- Oligonucleotides resuspension buffer (e.g. TE pH 8.0);
- TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems);
- Sense primer Class II A for 5'-CTC ACC CCY CTT GGT GA-3';
- Antisense primer Class II A rev 5'- GGA GRA TGT TGG CAG CAT T-3';
- Probe Class II A avir MGB 5'-FAM-CCT ATA AGG CGY CCC TGT YTC-MGB-3';
- Probe Class II A vir MGB probe 5'-Cy5-CCT AYA AAG CGT YTC TGY CTC C-MGB-3';
- Sense primer Class II B for 5'-GAR GCA TAY AAC AGA ACA-3';
- Antisense primer Class II B rev 5'-GTY GCA ACC CCR AGA GCT A-3';
- Probe Class II B avir MGB 5'-FAM-GAR ACA GGG ACG YCT TAT AGG-MGB-3';
- Probe Class II B vir MGBa 5'-Cy5-ARA CGC TTY ATA GGT GC-MGB-3';
- Probe Class II B vir MGBb 5'-Cy5-AAR CGY TTT RTA GGT GC-MGB-3';
- Sense primer Class I C for 5'-CMG GGA CAA TTA TCA TCA A-3';
- Antisense primer Class I C rev 5'-GGC TAC ACC TAA TGC GA-3';
- Probe Class I C MGB 5'-FAM-CAG GAG CGK TTG RTA GG-MGB-3'.

4.2. Equipment

- General molecular biology laboratory equipment and consumables;
- QuantStudio 5 Real-Time PCR System (Applied Biosystems) (or equivalent real-time PCR platform);
- QIAxcel Advanced system (Qiagen) (or equivalent electrophoresis and imaging systems for stained gels);
- 3130xl Genetic Analyzer (LifeTechnologies) (or equivalent sequencing system/service).

5. Procedure

5.1. Controls

To ensure test reliability, the controls listed in Table 1 must be included in each run and for all the designated RRT-PCR assays/targets.

Control Type	Definition
Positive template control (amplification control, PTC)	Sample containing a known amount of the target RNA and stored at ≤ -70°C, that is processed along with the samples starting from the RRT-PCR phase
Negative template control (NTC)	Sample containing all PCR reagents but no target RNA, that is processed along with the samples starting from the RRT-PCR phase

Table 1. Controls to ensure test reliability

This procedure is applied as a downstream method to samples which have tested positive for OAV-J by a screening molecular assay. In case this procedure is applied as a frontline method from a new sample aliquot, a negative process control (NPC) (i.e. sample containing no target organism that is processed along with the samples starting from the nucleic acids isolation phase), and a positive process control (PPC) (i.e. sample containing the target organism that is processed along with the samples starting from the nucleic acids isolation phase) can be used to assess the reliability of the analytical process.

5.2. Preparation of samples and storage

For samples preparation see SOP VIR 1000.

Clinical samples and viral isolates must be stored at refrigerated temperature (2-8°C) until the completion of the analysis. For long term conservation, store samples at ≤ -70°C.

Purified nucleic acids can be refrigerated for a few hours prior RRT-PCR, otherwise they must be stored at ≤ -70°C.

5.3. Isolation of nucleic acids

RNA can be isolated either by manual or automatic methods. The list of nucleic acids isolation systems validated at the IZSVe on different types of matrices is available in SOP VIR 1000.

5.4. Real-time RT-PCR

The preparation of the master mix for the RRT-PCR reactions has to be carried out in a clean dedicated area. The reagents should be kept refrigerated throughout the entire preparation phase. Probes should be protected from light. For the whole procedure, the use of filter tips and nuclease-free plastics is recommended.

Reaction mix preparation

Prepare a master mix volume sufficient for the number of samples to be tested in each run. The volumes reported in Tables 2-4 are per single reaction.

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	7.25
TaqMan Fast Virus 1-Step Master Mix	4X	1X	6.25
Sense primer Class II A for	10 µM	1 µM	2.5
Antisense primer Class II A rev	10 µM	1 µM	2.5
Probe Class II A avir MGB	10 µM	0.3 µM	0.75
Probe Class II A vir MGB probe	10 µM	0.3 µM	0.75
Master mix minus template			20
Template			5
Total reaction volume			25

Table 2. Volume and concentration of the reaction mix components for the RRT-PCR assay employing **SET A** oligonucleotides, targeting class II OAV-J of genotypes I-IV, IX-XI

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	6.875
TaqMan Fast Virus 1-Step Master Mix	4X	1X	6.25
Sense primer Class II B for	10 µM	1 µM	2.5
Antisense primer Class II B rev	10 µM	1 µM	2.5
Probe Class II B avir MGB	10 µM	0.25 µM	0.625
Probe Class II B vir MGBa	10 µM	0.25 µM	0.625
Probe Class II B vir MGBb	10 µM	0.25 µM	0.625
Master mix minus template			20
Template			5
Total reaction volume			25

Table 3. Volume and concentration of the reaction mix components for the RRT-PCR assay employing **SET B** oligonucleotides, targeting class II OAV-J of genotypes V-VIII, XII-XXI

Component	Initial concentration	Final concentration	µl per reaction
Nuclease-free water	-	-	8.625
TaqMan Fast Virus 1-Step Master Mix	4X	1X	6.25
Sense primer Class I C for	10 µM	0.9 µM	2.25
Antisense primer Class I C rev	10 µM	0.9 µM	2.25
Probe Class I C MGB	10 µM	0.25 µM	0.625
Master mix minus template			20

Template	5
Total reaction volume	25

Table 4. Volume and concentration of the reaction mix components for the RRT-PCR assay employing **SET C** oligonucleotides, targeting class I OAV-J

The master mix should be mixed thoroughly and 20 µl solution per sample must be pipetted in real-time PCR strip/plate.

Template must be added in a separate room. To minimize the risk of cross-contamination, it is recommended to add controls and samples in the following order: NTC, NPC (if used), nucleic acids isolated from diagnostic samples, PTC, PPC (if used). In case of NTC, 5 µl of nuclease-free water should be added.

Cycling conditions

Place the strip/plate in the real-time PCR apparatus. Set up the loading scheme as well as the thermal profile as per Tables 5 and 6, and select FAM and Cy5 compatible detection channels for fluorescence acquisition. Employ a ramp/rate of 1.6°C/sec.

Step	**Temperature and time**	**Repetitions**
Reverse transcription	50°C for 5 min	1
RT inactivation/initial denaturation	95°C for 20 sec	1
Denaturation	95°C for 30 sec	45
Annealing/extension (*)	60°C for 45 sec	

Table 5. Real-time RT-PCR thermal profile for the assay employing **SET A**. (*) Fluorescence acquisition has to be done during the annealing phase

Step	**Temperature and time**	**Repetitions**
Reverse transcription	50°C for 5 min	1
RT inactivation/initial denaturation	95°C for 20 sec	1
Denaturation	95°C for 30 sec	45
Annealing/extension (*)	55°C for 45 sec	

Table 6. Real-time RT-PCR thermal profile for the assay employing **SET B** and **SET C**. (*) Fluorescence acquisition has to be done during the annealing phase

5.5. Data analysis

Upon completion of the amplification reaction, amplification plots must be critically assessed. The baseline and the threshold can be set either automatically or manually. The threshold should be placed above the background fluorescence noise (c.ca 0.04 ΔRN), across the exponential phase of all the amplification curves (corresponding

to the early linear phase of the logarithmic view). The use of a PTC with a known target concentration can act as a calibrator to enable standardization of data analysis and an approximate estimation of the viral load.

5.6. PCR product detection

The presence of PCR products and their size analysis can be performed either by automatic (e.g. QIAxcel Advanced system, Qiagen) or manual (7% acrylamide gel or 2% agarose gel) electrophoresis systems, using appropriate DNA molecular markers. Expected size is 149-218 bp, depending on the assay.

5.7. Sanger sequencing

Sanger sequencing of the amplification product is highly recommended in the following cases: i) in primary ND outbreaks (i.e. the index case), ii) to interpret dubious RRT-PCRs results (Cq > 35), iii) in case of co-infection with virulent and avirulent OAV-J. The amplification products should be diluted 1:5 v/v with nuclease-free water before performing the sequencing reaction. Sequencing and sequence analysis should be carried out according to the methods and software in use at each laboratory.

6. Interpretation of results

6.1. Reliability of controls

Test reliability is assured if the controls yield the expected results, as reported in Table 7. In case of invalid results, the causative reason must be investigated and proper actions have to be taken.

Control	Expected Result	Action in case of invalid control
PPC (if used)	Increase in fluorescence from the FAM/Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the nucleic acids extraction
NPC (if used)	Absence of fluorescence increase from the FAM/Cy5 fluorophores, with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the nucleic acids extraction and check nucleic acids isolation reagents
PTC	Increase in fluorescence from the FAM/Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve at the expected Cq value	Repeat the analysis from the RRT-PCR and check the PTC stock
NTC	Absence of fluorescence increase from the FAM/Cy5 fluorophores, with no sigmoidal (or logarithmic) amplification curve	Repeat the analysis from the RRT-PCR and check RRT-PCR reagents

Table 7. Assessment of test reliability. Cq = quantification cycle

6.2. Diagnostic samples

Criteria for data interpretation of diagnostic samples and subsequent actions are reported in Table 8.

Result	Interpretation and action
Increase in fluorescence only from the FAM fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 35 for SET A, SET B and/or SET C	Positive for avirulent OAV-J. The amplification product can be subject to Sanger sequencing to determine the CS sequence of the F protein gene, if required
Increase in fluorescence only from the Cy5 fluorophore yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 35 for SET A and/or SET B	Positive for virulent OAV-J. The amplification product can be subject to Sanger sequencing to determine the CS sequence of the F protein gene, if required
Increase in fluorescence from both the FAM and Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve with Cq ≤ 35 for SET A, SET B and/or SET C	Positive for avirulent and virulent OAV-J. The amplification products must be subject to Sanger sequencing to determine the CS sequences of the F protein gene
Absence of fluorescence increase from the FAM and Cy5 fluorophores, with no sigmoidal (or logarithmic) amplification curve	Not typeable. Another method can be applied for the molecular pathotyping of OAV-J
Weak increase in fluorescence from the FAM and/or Cy5 fluorophores yielding a sigmoidal (or logarithmic) amplification curve with Cq > 35	Doubtful. The amplification product must be subject to Sanger sequencing to determine the CS sequence of the F protein gene for pathotype confirmation. In case Sanger sequencing can not be adopted, or yields poor quality sequences, the sample has to be considered as “Not typeable” and it is responsibility of the Head of the Laboratory to identify the actions to be taken

Table 8. Interpretation of real-time RT-PCR diagnostic results

6.3. OAV-J pathotype interpretation

The quality of chromatograms covering the F0 cleavage site should be sufficient to allow the correct sequence interpretation. The example in Figure 1 shows the chromatogram of two complementary sequences in opposite orientations, of acceptable quality (i.e. clear and high signal, no background noise, distinct peaks, unambiguous base calling). Contrarily, chromatograms showing high background, superimposed peaks and numerous ambiguous nucleotides are considered of poor quality and therefore unfit, and should require further RT-PCR and/or sequencing attempts.

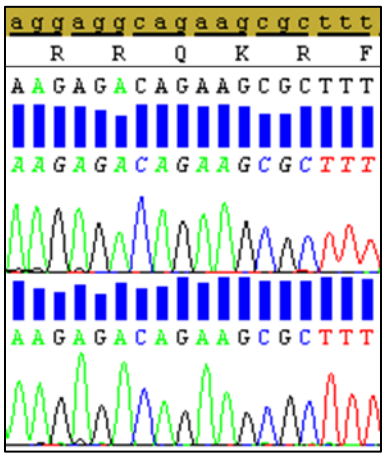

Figure 1. Chromatogram of a virulent OAV-J cleavage site

Nucleotide sequence data of acceptable quality result in a reliable translation into deduced amino acid sequence of an OAV-J F0 cleavage site that is therefore eligible for pathotype identification, according to the guidelines reported in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Chapter 3.3.10. Most OAV-J viruses that are pathogenic for chickens have the motif ^{112}R/K-R-Q/K/R-K/R-R^{116} at the C-terminus of the F2 protein and F^{117} at the N-terminus of the F1 protein, whereas the viruses of low virulence have the residues ^{112}G/E-K/R-Q-G/E-R^{116} and L^{117}. Some of the pigeon variant viruses (PPMV-1) have the sequence ^{112}G-R-Q/K-K-R-F^{117}, but give high ICPI values. Thus, at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 seem to be required to show virulence for chickens. However, some PPMV-1 may have virulent cleavage sites with variable ICPI values.

The size of the RRT-PCR amplification product is not suitable for reliable genotype determination, and RT-PCR assays or NGS-based methods that permit to obtain the sequence of a larger fragment of the fusion protein gene, should be preferred.

7. Characteristics of the method

This method was validated at the IZSVe according to the ISO/IEC 17025, employing OAV-J samples, selected avian viruses and bacteria available at the IZSVe repository, as well as materials, equipment and procedures as described above. The validation dossier is accessible upon request by contacting eurl.ai.nd.secretariat@izsvenezie.it

The method usually yields positive results for samples with low Cq values (≤ 30) by L-gene real time RT-PCR screening method. However, sensitivity can be strain-dependent, varies among genotypes, and might be affected by poor RNA quality, low viral load and the presence of PCR inhibitors. The shift of Cq values compared to that of screening assays can be remarkable (up to 10 cycles).

The method requires optimization of RRT-PCR amplification conditions in case different reagents and instruments are adopted. Any modification to this SOP by third party laboratories should be supported by proper validation data assessing that the method is still fit-for-purpose.